Specific recognition of non-denatured nitrite-oxidizing system of *Nitrospira moscoviensis* by monoclonal antibody Hyb 153-3

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Abstract:

The objective of this research project is to develop a rapid molecular method for monitoring nitrification in a wastewater reactor. In the developed method, a monoclonal antibody (Hyb 153-3) was used because it can specifically recognize non-denatured enzymes responsible for nitrite oxidation in *Nitrobacter* and *Nitrospira*. The recognition of these enzymes under non-denatured conditions can significantly simplify the procedures of future immunoassays for environmental samples collected from various natural and engineered systems. This paper presents the ability of the selected Hyb 153-3 antibody to recognize the non-denatured form of the nitrite-oxidizing enzyme of *Nitrospira* in an aqueous phase as well as when the enzyme has been immobilized on a solid surface. Copyright © 2005 Society of Chemical Industry

Keywords: Hyb 153-3 antibody | autotrophic nitrifying bacteria | biological nutrient removal | chemistry | biochemistry

Article:

INTRODUCTION

The activity of autotrophic nitrifying bacteria is an important factor controlling wastewater biological nutrient removal (BNR) processes. With respect to nitrite oxidation, prior BNR processes were engineered based primarily on our understanding of *Nitrobacter* species, the most studied nitrite-oxidizing bacteria. Yet recent studies revealed that *Nitrospira* spp. might be the dominant autotrophic nitrite-oxidizing bacteria in many natural and engineered systems.1–4

Although immunological assays can be quite effective in quantifying target bacteria and sometimes even the activity stage of the cells, an assay may become too limited serologically if the antibody (Ab) employed can recognize only one type of bacteria. For a wastewater BNR system, an assessment of overall nitrite-oxidation activities should measure both *Nitrobacter* and *Nitrospira* spp. Bartosch *et al.*5 reported that the monoclonal antibody (MAb) Hyb 153-3 (Mouse IgG_1) can recognize the nitrite-oxidizing system (NOS) of the γ- and δ-*Proteobacteria* and *Nitrospira* phylum, as well as the nitrite oxidoreductase (NOR) of the α-*Proteobacteria*. Hyb 153-3 appeared to react with sites on the β-subunit of NOR and NOS, which makes it an ideal antibody for application to BNR processes.

Previous studies on Hyb 153-3 reactivity for the NOS of *Nitrospira* spp. all reported assay procedures using denatured NOS.5–8 Our study was designed to evaluate the recognition of non-denatured NOS of *Nitrospira* spp. by Hyb 153-3. Such a recognition property can significantly simplify future immunoassay procedures developed for environmental samples collected from various natural and engineered BNR systems.

EXPERIMENTAL DESIGN AND METHODS

Cell culture of *Nitrospira moscoviensis* was provided by Dr Eva Spieck of Universität Hamburg, Germany. The cell extract was prepared by sonicating the *N. moscoviensis* cells on ice for 45 min, and the cellular debris was removed by brief centrifugation at 1000 × *g* for 15 s. To label the cell extract protein, biotinamidohexanoic acid was selected. The labeling reaction was performed by adding 0.9 pmol of *N*-hydroxysuccinimide (NHS) ester of biotinamidohexanoic acid per µg total protein (measured by Pierce Micro-BCA™ Assay [Pierce Biotechnology Inc., Rockford, IL]). The mixture was incubated at 25 °C for 3 h. The reaction was quenched by adding 1 µL of 10× Tris buffered saline (10 × TBS) (0.5 mol L^{-1} Tris-free base, 1.55 mol L^{-1} NaCl) per 100 pmol NHS–biotinamidohexanoic acid added. Any free biotin label was removed by filtering the labeled extract through a 10 kDa molecular weight cut-off (MWCO) Microcon® filter (Millipore Corp., Billerica, MA). Proteinase inhibitor (Roche Inhibitor Cocktail Tablets, EDTA-Free) was added (Diagnostics Corp., Indianapolis, IN), and the concentrated extract was diluted with phosphate buffered saline (PBS) (10 mmol L^{-1} phosphate buffer pH 7.4, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl). Labeled extract was stored at − 20 °C.

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting:

The recognition of Hyb 153-3 with non-denatured cell extract was carried out via immunoblotting. Cell extract (10 µg) was loaded into a 10% Tris–HCl polyacrylamide gel (8.6 × 6.8 cm). The sodium dodecyl sulfate (SDS) denaturant in the gel running buffer was omitted, and the cell extract was not subjected to any heat treatment or denaturants prior to loading. Replicates (n = 3) were loaded into the same gel. The extract was electrophoretically separated by applying 7.35 V cm^{-1} for 15 min and followed by 14.7 V cm^{-1} for approximately 75 min. The gel was then cut lengthwise into two portions. The separated extract from one portion of the gel was transferred onto a nitrocellulose membrane at 30 V overnight. The band that corresponded to the recognition of non-denatured β-NOS by Hyb 153-3 was developed using the BIO-RAD Goat-

anti-Mouse Opti-4CN™ Detection Kit (BIO-RAD Laboratories Inc., Hercules, CA) and was observed in the range between 150 and 250 kDa. To confirm the identity of the observed band, the region corresponding to the observed molecular weight range was excised from the other gel portion and masticated. The masticated gel was incubated in the presence of a Tris buffered glycine with SDS buffer solution (25 mmol L^{-1} Tris buffer pH 8.3, 192 mmol L^{-1} glycine, 0.1% w/v SDS) for 24 h at 4 °C and then added to a 0.45 µm spin column (Pall Corp., East Hills, NY). The eluent from the spin column was further analyzed by denaturing PAGE, followed by the same immunoblotting procedure as described above.

Direct enzyme-linked immunosorbent assay

A direct enzyme-linked immunosorbent assay (ELISA) was performed to verify Hyb 153-3 recognition of non-denatured β-NOS deposited on a fixed surface. Purified NOS is unavailable, therefore, all ELISA work was performed with cell extract of *N. moscoviensis*. A 50 µL volume of cell extract diluted in 50 mmol L^{-1} carbonate buffer solution (CBS) at pH 9.6 was pipetted into each microtiter well (Nunc™ 96-well Maxisorp™; Nalgene Nunc Intl., Rochester, NY) and immobilized on the microplate surface by incubating at 37 °C for 1 h. The working range of coating concentrations had been determined to span from 0.175 µg mL^{-1} to 2.92 µg mL^{-1} cell extract as total protein. The microtiter wells were washed six times with a phosphate buffered saline with Tween® 20 (PBST) washing buffer (10 mmol L^{-1}phosphate buffer pH 7.4, 138 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, and 0.05% Tween® 20) to remove unbound cell extract. The microtiter wells were blocked with 300 µL of Pierce TBS SuperBlock® (Pierce Biotechnology Inc., Rockford, IL) for 5 min at 25 °C. The blocking was repeated twice with fresh blocking solution for 5 min and then 30 min. The wells were then washed six times with PBST washing buffer. A 50 µL volume of 1 µg mL^{-1} Hyb 153-3 MAb diluted in PBS buffer was added to each well and incubated at 25 °C for 1h. The unbound Hyb 153-3 was removed, and the wells were washed six times with PBST. The immunocomplexes formed between non-denatured β-NOS and Hyb 153-3 in the wells were detected by incubating with 50 µL of 0.33 µg mL^{-1} goat-anti-mouse alkaline phosphatase (GAM-AP) conjugate diluted in Tris buffered saline (50 mmol L^{-1} Tris buffer pH 8.0, 2.7 mmol L^{-1} KCl, 137 mmol L^{-1} NaCl). The plate was incubated for 1 h at 25 °C, after which the GAM-AP solution was removed and the wells were washed ten times with PBST. A 5.0 mmol L^{-1}solution of 4-methylumbelliferyl phosphate disodium salt trihydrate was freshly prepared in TBS solution, and 50 µL was added to each well as substrate. Finally, the enzymatic reaction was allowed to proceed for 40 min at 25 °C, after which the fluorescence signal (335 nm excitation, 444 nm emission) was measured by a Thermo-Orion Fluoroskan Ascent FL plate reader (Thermo-Orion Inc., Waltham, MA). Omitting cell extract and/or Hyb 153-3 MAb were used as negative controls to verify the specificity of GAM-AP and Hyb 153-3.

Cell extract processing for background attenuation

Based on prior experimental results, processing procedures were developed to attenuate the potential high background signal resulting from non-specific interactions of biotinylated cell extract with the microtiter well surface. Cell extract is diluted to 60 µg mL^{-1} total protein

concentration with PBS. Eight consecutive wells in a microtiter plate are coated with 50 µL of 1 µg mL^{-1} Anti-BSA Clone BSA-33 (not reactive towards cell extract) MAb (Sigma-Aldrich Inc., St Louis, MO) in CBS. Incubation of the well coating solution, washing, blocking, and washing proceeded as described for the direct ELISA. Next, 50 µL of cell extract is introduced to the first well and incubated for 30 min at 25 °C, while 50 µL of PBS is added to each of the other wells. After 30 min, the PBS is drawn off from the adjacent well (well No. 2), and the cell extract in the first well is then transferred to this adjacent well. The incubation-transfer process is repeated seven times, after which the cell extract is drawn off the final well, concentrated via centrifugation in a 10 kDa MWCO MicroCon® spin column at 12 000 × *g* for 30 min, and then diluted to the pre-filtering volume with PBS.

Immunological reaction in aqueous phase

In a microcentrifuge tube, biotinylated cell extract was incubated with an estimated equal molar quantity of unlabeled Hyb 153-3 in PBS for 1 h at 25 °C to form an immunocomplex between β-NOS and Hyb 153-3. Recombinant Protein G (Rockland Immunochemical Gilbertsville, PA) was immobilized on each microtiter well by incubating 50 µL of 1 µg mL^{-1} Protein G in CBS, pH 9.6, for 1 h at 37 °C. Well washing and blocking were all carried out as described for the direct ELISA. The immunocomplex solution was then added to the microtiter wells, followed by incubation for 1 h at 25 °C. After the incubation, the wells were washed eight times with PBST. Streptavidin–AP conjugate addition, well washing, substrate addition, and signal development all proceeded as described for the direct ELISA. Positive controls were created by capturing only biotinylated Hyb 153-3 on a Protein G-coated microtiter well. The background signal was measured by omitting any labeled Hyb 153-3 or immunocomplex in a Protein G-coated well.

RESULTS

The specific recognition of the non-denatured NOS by the Hyb 153-3 antibody was demonstrated in this study under two different experimental approaches: (1) the β-NOS immobilized on a solid surface (e.g. a nitrocellulose membrane or microtiter plate), and (2) in an aqueous phase to avoid potential structural alterations of the protein.

Using denaturing PAGE, specific Hyb 153-3 reactivity with β-NOS was initially determined at 46kDa, agreeing with size of the β-NOS subunit previously reported by Bartosch *et al.*5 In contrast, the band that was identified as the β-NOS subunit by non-denaturing PAGE was comparatively broad, as shown in Fig. 1(a). In non-denatured cell extract, the membrane-bound NOS molecules likely remained affixed to various-sized membrane fragments, resulting in the observed band broadening (Fig. 1(a)). However, by analyzing the elution from a non-denaturing gel slice excised from the same molecular weight region of 150 kDa to 250 kDa where the broad, non-denatured band appeared (Fig. 1(a)), specific Hyb 153-3 reactivity with β-NOS was clearly observed at 46 kDa using denaturing PAGE (Fig. 1(b)).

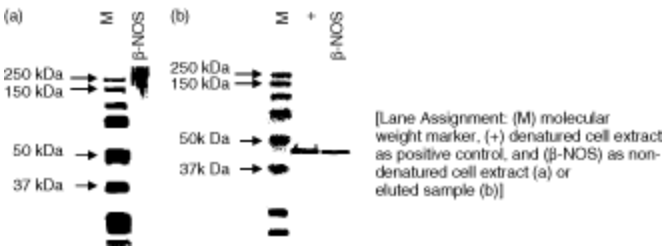

Figure 1. (a) Immunoblot of non-denatured cell extract, separated by non-denaturing PAGE. (b) Immunoblot of β-NOS eluted from the excised gel slice, separated by denaturing PAGE. (M = molecular markers, and '+' = positive control of β-NOS).

A semi-quantitative, direct ELISA calibration was performed with various dilutions of non-denatured cell extract (measured as total protein by Pierce Micro-BCA™ Assay) immobilized on the microtiter wells. By adding a fixed amount of Hyb 153-3 to the wells, a highly linear correlation ($r^2 = 0.99$) between cell extract and fluorescence signal was observed (Fig. 2), suggesting specific recognition of the deposited β-NOS by Hyb 153-3. The ELISA results, coupled with positive immunoblotting presented above, confirmed the recognition of non-denatured β-NOS by Hyb 153-3 when the antigen is immobilized on a solid surface.

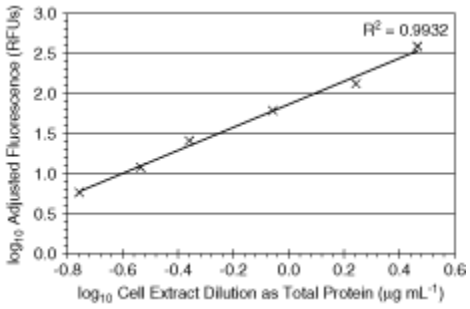

Figure 2. Direct ELISA calibration. Adjusted fluorescence is measured fluorescence minus background. RFU, relative fluorescent unit.

To demonstrate recognition of non-denatured β-NOS without immobilization on a solid surface, the Hyb 153-3 reaction with biotinylated cell extract was carried out in the aqueous phase. Protein G, deposited on a microtiter well, was then used to capture the F_C region of Hyb 153-3 that formed the immunocomplex with β-NOS. Results from the Protein G capture of the immunocomplex between Hyb 153-3 and labeled cell extract are shown in Fig. 3, where fluorescent signals are presented as the average of triplicate wells with error bars of one standard deviation. The signal (317 ± 9.5 RFU) detected in wells incubated with the immunocomplexes indicates a positive capture of immunocomplexes by the Protein G. The positive signal statistically exceeds the background signal (269.9 ± 5.9 RFU), which was detected in wells where only labeled cell extract was added. The background signal was caused by non-specific binding of labeled proteins to the wells (data not shown). Specificity of the Hyb 153-3 MAb to β-NOS and an absence of cross-reactivity were previously demonstrated by Bartosch *et al.*5 The

positive detection was also confirmed when compared with the statistically similar signal (327.9 ± 26 RFU) detected in positive control wells. For positive control wells, no cell extract was added and the same amount of biotinylated Hyb 153-3 was added as in the wells for immunocomplex capture.

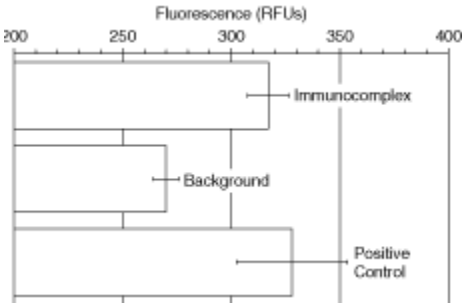

Figure 3. Detection of Hyb 153-3/β-NOS immunocomplex formed in aqueous phase.

CONCLUSIONS

The linear result observed in the direct ELISA coupled with the immunoblotting results indicate a high degree of specificity for Hyb 153-3 detection of non-denatured β-NOS deposited on a solid surface. However, the true benefit to ultimate development of quantitative assays will come if the recognition can also occur in the non-denatured, aqueous phase, thereby significantly simplifying assay procedures. The statistically similar signal observed in immunocomplex capture wells and positive control wells showed that immunoreaction in an aqueous phase and the subsequent capturing of immunocomplexes can be carried out with a fairly high efficiency under the conditions tested in this study. The recognition of non-denatured β-NOS by Hyb 153-3, and the reporting assay conditions are valuable to the development of immunoassays for monitoring nitrification in natural and engineered environmental systems.

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